Multiple mating increases cocoon hatching success in the earthworm *Eisenia andrei* (Oligochaeta: Lumbricidae)

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In simultaneous hermaphrodites with reciprocal mating, multiple mating may be a male strategy that conflicts with female interests, and therefore an intra-individual sexual conflict regarding the number of matings may be expected. The evolutionary outcome of this sexual conflict will depend on the costs and benefits that extra mating entails for each sexual function. In the present study, we investigated the costs and benefits of multiple mating on cocoon number, cocoon mass, and cocoon hatching success in the redworm *Eisenia andrei*, a simultaneous hermaphrodite with reciprocal insemination, by manipulating the number of matings with different partners. We did not detect any reduction in the female reproductive output (number and mass of cocoons) with increasing number of mating partners. However, we found that multiple mating showed benefits for female reproduction that increased the hatching success of the cocoons. This effect may be a result of increased quantity and/or diversity of sperm in the spermathecae of multiple mated earthworms. Further studies are required to clarify the mechanism underlying the increased cocoon hatching success when redworms engage in multiple matings. © 2012 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2012, **107**, 175–181.

ADDITIONAL KEYWORDS: genetic benefits – mating costs – polyandry – sexual conflict – simultaneous hermaphrodites – sperm depletion.

INTRODUCTION

Many animals copulate more than is necessary for simple fertilization (Birkhead & Møller, 1998; Arnqvist & Nilsson, 2000). In gonochoristic animals, males typically increase their reproductive success by mating with many females (Bateman, 1948; Trivers, 1972). In addition, repeated mating with the same female may increase insemination success and hence the paternity of males (Laird, Gwynne & Andrade, 2004). Nevertheless, multiple matings may not be advantageous for females because one copulation (or only a few) often provides enough sperm to fertilize all of a female's eggs (Arnqvist & Nilsson, 2000) and therefore further copulations imply a waste of time and energy (Daly, 1978; Thornhill & Alcock, 1983). Thus, multiple mating may be a male strategy that conflicts with female interests (Stockley, 1997), although some benefits of multiple mating may also occur from a female perspective (Arnqvist & Nilsson, 2000; Jennions & Petrie, 2000; Neff & Pitcher, 2005; Simmons, 2005).

In simultaneously hermaphroditic animals, empirical results also suggest stronger selection to gain mating partners in male rather than female function (Anthes *et al.*, 2010; Pélissié, Jarne & David, 2011). For example, in the freshwater snail *Physa acuta* Draparnaud, 1801, the number of matings increases male reproductive success more than that of females (Pélissié *et al.*, 2011). Simultaneous hermaphrodites have male and female reproductive organs that function at the same time in the same individual, and therefore an intra-individual conflict of interest between both sexual functions ('sexual conflict') may be expected with respect to the optimal number of matings, (Parker, 1979, 2006; Arnqvist &

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Rowe, 2005). In the particular case of species with reciprocal mating (i.e. individuals copulate in both sexual roles), independent optimization of male and female mating frequency is especially restricted (Anthes *et al*., 2010).

Mating usually entails remarkable energy expenses (Daly, 1978; Thornhill & Alcock, 1983) as a result of the metabolic costs of sexual behaviour (Watson, Arnqvist & Stallman, 1998) and a reduction in foraging efficiency (Stone, 1995; Takahashi & Watanabe, 2010). Thus, from a female perspective, multiple mating may reduce the energy available for egg production. For example, in the sea slug *Chelidonura sandrana* Rudman, 1973, female function suffers from a linear decrease in fecundity with increasing mating rate (Sprenger *et al*., 2008b). Moreover, simultaneous hermaphrodites probably have a limited reproductive energy budget. Therefore, a trade-off between male and female function ('sex-allocation trade-off') is likely to occur (Charnov, 1982; Schärer, 2009). For example, in the flatworm *Macrostomum* sp., an increase in the mating group size results in larger testes and smaller ovaries (Schärer, Sandner & Michiels, 2005).

By contrast, several benefits for female reproduction have been proposed to explain multiple mating from a female perspective. For example, multiple mating may increase the amount of sperm available to fertilize the eggs, thus reducing the risk of sperm limitation (sperm quantity benefits; Levitan & Petersen, 1995; Fjerdingstad & Boomsma, 1998; MacDiarmid & Butler, 1999). Multiple mating may also enhance egg production in those species in which males provide females with nutritious ejaculates or nuptial gifts (Eberhard, 1996; Vahed, 1998; Arnqvist & Nilsson, 2000). Finally, it has been proposed that copulation with several males (polyandry) can provided genetic benefits for females as consequence of increased sperm diversity (Jennions & Petrie, 2000; Neff & Pitcher, 2005; Simmons, 2005). By mating with different males, females have the potential to utilize postcopulatory mechanisms (i.e. sperm competition and/or cryptic female choice) to acquire good genes for their offspring (Fisher *et al*., 2006), or to reduce the risk of syngamy with sperm carrying incompatible genes (Zeh & Zeh, 1996, 1997) or damaged genes (Radwan, 2003; Velando, Torres & Alonso-Alvarez, 2008b). Polyandry may also reduce the risk of infertility (Simmons, 2005; Marshall & Evans, 2007).

In the present study, we investigated the effect of multiple mating on female reproduction in the redworm *Eisenia andrei* (Bouche, 1972), a simultaneous hermaphrodite with reciprocal insemination. We experimentally manipulated the number of mating partners by sequentially mating focal earthworms with one to six mating partners. We then analyzed the effect of this treatment on the number and mass of cocoons and on cocoon hatching success. Cocoons are capsules comprising a wall of chitin-like material containing several fertilized eggs as well as the albumin required to feed embryos during development.

Redworms usually live in dense populations in which multiple mating is common (Monroy *et al*., 2003). Copulation is preceded by a prolonged courtship that involves short repeated touches between partners (Grove & Cowley, 1926). During copulation, sperm are exchanged simultaneously and reciprocally, and stored in two pairs of spermathecae until cocoon production. Redworms are unable to digest the sperm in their spermathecae (Richards & Fleming, 1982), thereby reducing the possibility of nutritional benefits via sperm digestion. Sperm from different mates is probably mixed in the spermathecae (Velando, Eiroa & Domínguez, 2008a), enabling sperm competition (Parker, 1998) and/or cryptic female choice (Thornhill & Alcock, 1983).

If mating is costly, we expect a reduction in the female reproductive output (number and/or mass of cocoons) as the number of mating partners increases. Our experimental design does not allow differentiation between sperm diversity and sperm quantity effects but, if multiple mating increases the genetic quality of the progeny and/or the fertilization success of the eggs, we expect an increase in the cocoon hatching success.

MATERIAL AND METHODS

COLLECTION AND MAINTENANCE

One hundred and eighty hatchlings of *E. andrei* were obtained from our laboratory cultures, which contain more than 10 000 individuals. The cultured earthworms mainly originate from a population established in a manure heap close to the University of Vigo (northwest Spain; 42°9′N, 8°41′W). However, we periodically add individuals from other populations aiming to maintain genetic diversity of the stock population. Hatchlings were isolated in individual plastic Petri dishes to ensure that the specimens were virgins at the time of mating. Petri dishes were filled with vermicompost as a breeding medium and kept in darkness in an incubator at 18–22 °C. The earthworms were fed with horse manure supplied *ad libitum*, and water was added to avoid dehydration of the medium. Earthworms began to reach sexual maturity, as indicated by the development of the tubercula pubertatis and clitellum, at the age of 1 month. However, the earthworms were not mated until they were 2 months old, aiming to ensure that they were all fully mature.

EXPERIMENTAL SET-UP

Forty mature virgin earthworms (focal earthworms) were randomly assigned to four experimental groups (ten per group) and were sequentially mated with one, two, four or six virgin partners. The initial body mass of focal earthworms did not differ among experimental groups (mean ± SE: 0.66 ± 0.048 g, 0.57 ± 0.034 g, 0.62 ± 0.048 g, 0.66 ± 0.038 g; analysis of variance: $F_{3,34} = 0.97$, $P = 0.42$). Earthworms were marked with a tiny light burn on different segments behind the clitellum, so that they could be recognized after mating.

We placed each focal earthworm with a virgin partner in a Petri dish with vermicompost and horse manure. During mating, both earthworms are attached in an inverse position at their ventral sides and sperm transfer takes place from the male pores up the spermathecae of the partner. All the pairs were examined daily until the appearance of spermatophores, indicative of recent mating (Monroy *et al*., 2003). To control handling stress, irrespective of the completion of treatment, earthworms were daily examined until all focal earthworms completed the assigned matings. After mating, the partner was removed. The process was repeated until the total number of mating partners assigned in each experimental group was reached. Mating trials were completed within 1 and 8 days (mean ± SE: 3.78 ± 0.88 days) in the one-partner group, 2 and 10 days (7.11 ± 0.81 days) in the two-partner group, 8 and 18 days (13.40 ± 1.09 days) in the four-partner group, and 20 and 35 days (25.11 ± 1.61 days) in the six-partner group. One focal earthworm from the six-partner group did not complete the mating trials and was excluded from the analysis.

After mating, the focal earthworms were isolated in Petri dishes until week 13, after the beginning of mating (week 1). Focal earthworms were weighed at week 1 and week 13 to calculate the change in body mass. Redworms typically begin to lay cocoons 48 h after copulation, at a rate of approximately three cocoons per week (Domínguez & Edwards, 2010). Cocoons laid by focal earthworms during the 13 weeks of the experiment were collected, weighed, and placed in individual wells of microplates. Microplate wells were covered with wet cotton to maintain the humidity, and then covered with a layer of Parafilm (Pechiney Plastic Packaging Company) in which small holes were made to reduce evaporation, at the same time as enabling air exchange. The Parafilm also prevented mixing between hatchlings from different cocoons. Cocoons were incubated in darkness in an incubator, at 18–22 °C, until hatching. (18–26 days after lay). In redworms, two or three hatchlings usually hatch per cocoon (Domínguez & Edwards, 2010). We considered that a cocoon has hatched when at least one hatchling appeared. Cocoon hatchability was calculated as the proportion of cocoons that hatched.

After each mating trial, partners of the focal earthworms were anaesthetized with chloroform and fixed in a 1 : 1 mixture of 4% formaldehyde : 96% ethanol and preserved with 4% formaldehyde in plastic tubes until dissection. To estimate sperm donated by focal earthworms, the four spermathecae of partners were removed under a stereomicroscope. The spermathecae were placed on a glass slide and compressed with a cover-slip to a uniform thickness of 71 μm: this was achieved by placing a copper wire of the same diameter between the slide and the coverslip. Each spermatheca was photographed with a digital camera (DXM1200F; Nikon). The sperm area in each spermatheca was measured by use of image analysis software (analySIS, Soft Imaging System GmbH). The sperm volume was estimated as the sperm area multiplied by 71 μm. The total volume of sperm delivered by focal earthworms in each treatment was calculated as the sum of the total volume of sperm in the spemathecae of all partners.

STATISTICAL ANALYSIS

One focal earthworm (from the one-partner group) did not lay cocoons, and another (from the two-partner group) died during the laying period; these earthworms were not included in the analyses. Sample size was nine in the one, two and six-partner groups and ten in the four-partner group. The effect of the number of mating partners on the number of cocoons produced was analyzed by a generalized linear model (GLM) with Poisson distribution and log-link function. The effect of the mating treatment on the mean cocoon mass was analyzed by a linear model (LM). The latter two analyses were applied to the total number of cocoons produced during the 13 weeks of the experiment (including those laid before the completion of treatments) to control any effect of reproductive effort and to study the effect of multiple mating on total female reproductive output. The effect of the mating treatment on cocoon hatching success (hatched cocoons as numerator and cocoons laid as denominator) was analyzed by a GLM with binomial distribution and logit function. This analysis was applied to two data subsets: one considering cocoons laid after all focal earthworms have completed their mating trials (i.e. after the fifth week) and second considering all cocoons laid over 8 weeks after each focal have completed its mating treatment. The first analysis standardizes to control laying order and the second standardizes by the time delay subsequent to the last mating. Any differences in the change of

earthworm body mass between the experimental groups were also analyzed by a LM. All models included the number of mating partners as a fixed factor and the initial earthworm body mass as a covariate, as well as its interaction.

Sensu Whittingham *et al*. (2006), full models including nonsignificant main effects were reported but excluding nonsignificant interactions (Engqvist, 2005). The effect of the number of mating partners on the total volume of sperm donated was analyzed by regression analysis. Curvilinear effects (linear, quadratic, cubic, and exponential) of the number of mating partners on total donated sperm were compared. In the six-partner groups, we also analyzed the effect of mating order on the volume of donated sperm to each partner by a linear mixed model, including focal identity as subject, the mating order as a fixed factor, and the focal and partner body mass as covariates. In GLMs, overdispersion was corrected by the Pearson scaled parameter when necessary. In LMs, data were checked for normality and homocedasticity by Kolmogorov–Smirnov and Levene's tests, respectively. Data are expressed as the mean ± SE. Analyses were performed using SPSS, version 16.0 (SPSS Inc.).

RESULTS

The number of cocoons laid by focal earthworms did not differ among experimental groups (GLM: $\chi^2 = 3.50$, d.f. = 3, $P = 0.32$), although it was positively related to initial body mass ($\chi^2 = 4.97$, d.f. = 1, $P = 0.026$). Mean cocoon mass was not affected by the number of mating partners (LM: $F_{3,32} = 1.05$, $P = 0.38$) but was positively correlated with initial body mass ($F_{1,32} = 35.02$, $P < 0.001$).

When we considered the cocoons laid after the fifth week, cocoon hatching success differed significantly between the different mating treatments (GLM: $\chi^2 = 8.07$, d.f. = 3, $P = 0.045$; Fig. 1). Interestingly, this effect was a result of the lower hatching success in the one-partner group compared to the multiple-partner groups (GLM: $\chi^2 = 8.52$, d.f. = 1, $P = 0.004$; Fig. 1). When we considered the cocoons laid after the end of the mating treatment, experimental treatments also differ in cocoon hatching success, although this difference was marginally significant (GLM: $\chi^2 = 7.61$, d.f. = 3, $P = 0.055$). Similarly, in this data set one-partner group showed lower hatching success compared to multiple-partner groups (GLM: $\chi^2 = 7.75$, d.f. = 1, $P = 0.005$). Cocoon hatching success was not related to the initial body mass of focal earthworms (cocoons laid after the fifth week: $\chi^2 = 0.59$, d.f. = 1, $P = 0.44$; cocoons laid after the last mating: $\chi^2 = 0.41$, d.f. = 1, $P = 0.52$).

The number of mating events did not affect body mass change of focal earthworms at the end of the
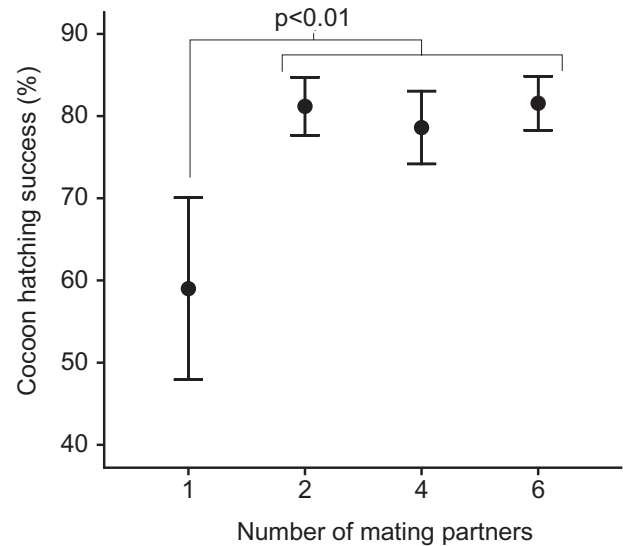

Figure 1. Mean ± SE cocoon hatching success of redworms in relation to the number of mating partners. Cocoon hatching success was expressed as the proportion of cocoons that hatch. The statistical significance of the difference between a one-partner and multiple-partner group is shown.

experimental period (LM: $F_{3,32} = 0.78$, $P = 0.51$; initial body mass: $F_{1,32} = 0.21$, $P = 0.65$). The total volume of sperm donated by focal earthworms increased with the number of mating partners ($F_{1,35} = 101.73$, $P < 0.001$), and the linear term was the relationship that best fit the data ($r_{37} = 0.86$, $y = 0.050 + 0.078x$). When we analyzed the six-partner group, mating order did not affect the volume of sperm donated to each partner ($F_{5,39.2} = 1.54$, $P = 0.20$). Neither focal, nor partner body mass affected the volume of donated sperm ($F_{1,6.99} = 2.21$, $P = 0.18$; $F_{1,42.67} = 0.81$, $P = 0.37$).

DISCUSSION

By contrast to our expectations, we did not observe any reduction in the female reproductive output with an increasing number of mating partners in the present study. On the other hand, the hatching success of cocoons laid by earthworms that had received sperm from multiple partners was higher than that of cocoons laid by earthworms mated with only one partner. The results suggest that, in redworms, multiple mating may have negligible costs, although it may also have benefits for female reproduction. Finally, we found that the total volume of donated sperm increased linearity with the number of mating partners. Therefore, focal earthworms did not suffer from autosperm depletion at least after six copulations. Indeed, in the six partner-group, earthworms transferred a similar amount of sperm to each partner.

Although it has been suggested that mating is energetically costly in redworms (Aira *et al.*, 2007; Velando, Eiroa & Domínguez, 2008a), in the present study, cocoon number and cocoon size were not reduced by multiple mating. Redworms may increase the investment in female reproduction when mated with several partners, as a consequence of maternal effects (Simmons, 2005; Sprenger *et al.*, 2010) or manipulative effects of the partners (Charnov, 1979; Koene, Sundermann & Michiels, 2002), which might mask the cost of multiple mating. On the other hand, life-history trade-offs may be obscured when organisms are cultured under *ad libitum* food conditions because high values of many life-history functions could be reached (Bell & Koufopanou, 1986; Schärer & Ladurner, 2003). Finally, our results could be explained if energy expenses of mating trade-off with other life-history traits different from female reproductive output, such as growth of indeterminate growers (Yund, Marcum & Stewart-Savage, 1997; Koene & Ter Maat, 2004; Aira *et al.*, 2007). However, we did not find any effect of the experimental treatment on body mass of focal earthworms, suggesting a similar residual reproductive value among earthworms of the different experimental treatments and hence a similar reproductive investment (i.e. reproductive effort in relation to the ability to invest in future reproduction; Trivers, 1972).

Interestingly, we found that mating with at least two different partners is beneficial for female reproduction in the redworm. The increased hatching success of cocoons laid by earthworms that have mated with multiple partners may be the result of an increased allocation of resources in cocoons as a consequence of maternal effects (Simmons, 2005; Sprenger *et al.*, 2010) or manipulative effects of partners (Charnov, 1979; Koene *et al.*, 2002). However, as already noted, in the present study, cocoon mass was similar among the experimental groups. Therefore, with the present evidence, the increased cocoon hatchability observed in multiple mating groups was probably not a result of an increased allocation of resources in cocoons.

The higher cocoon hatching success observed when earthworms mate with more than one partner may be attributed to sperm diversity and/or sperm quantity effects. High sperm diversity in the spermathecae of polyandric earthworms may lead, by postcopulatory mechanisms (i.e. sperm competition and/or cryptic female choice), to the acquisition of good genes for their offspring (Fisher *et al.*, 2006) or the avoidance of syngamy with sperm carrying incompatible genes (Zeh & Zeh, 1996, 1997) or damaged genes (Radwan, 2003; Velando *et al.*, 2008b). However, under the good genes hypothesis, differences between the two-, four- and six-partner groups may also be expected, although this was not the case. Our results may also be explained by an increased sperm quantity. Single-mated earthworms may run short of allosperm to fertilize their eggs and two matings may be sufficient to avoid sperm limitation. Finally, variability in cocoon hatching success was larger in the single-partner treatment than in the other experimental treatments because some single-mated earthworms had a very low hatching success. This may be indicative that some partners failed to donate sufficient amounts of sperm and also that receivers were allosperm limited.

An interesting result was that redworms transferred a similar amount of sperm to six successive partners. Furthermore, the time to mate did not differ among consecutive copulations. Taken together, these results indicate that autosperm is not depleted at least after six copulations. In redworms, germinal cells are continuously produced by testis, they continue their development in seminal vesicles, and, finally, mature spermatozoa are stored in the male funnels that lead sperm into *vasa deferentia* (Jamieson, 1981). Therefore, redworms probably replenish their reservoirs of mature spermatozoa among consecutive copulations.

In conclusion, we found no costs of multiple mating in the female reproductive output of redworms. By contrast, multiple mating increased the cocoon hatching success (i.e. it was beneficial for female reproduction). Male and female interests regarding the number of matings may be more similar than previously assumed (Anthes *et al.*, 2010), at least in redworms, and the promiscuity observed in this species (Monroy *et al.*, 2003) may be driven not only by male function (Bateman, 1948; Trivers, 1972), but also by female function. There are several possible mechanisms explaining the increased viability of cocoons produced by multiple-mated redworms. Further experiments that manipulate the degree of polyandry at the same time as controlling mating frequency (Sprenger, Anthes & Michiels, 2008a) are required to distinguish between sperm diversity and sperm quantity effects.

ACKNOWLEDGEMENTS

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